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Long-term persistence and bacterial transformation potential of transplastomic plant DNA in soil

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Abstract

The long-term physical persistence and biological activity of transplastomic plant DNA (transgenes contained in the chloroplast genome) either purified and added to soil or naturally released by decaying tobacco leaves in soil was determined. Soil microcosms were amended with transplastomic tobacco leaves or purified plant DNA and incubated for up to 4 years. Total DNA was extracted from soil and the number of transgenes (*aadA*, which confers resistance to both spectinomycin and streptomycin) was quantified by quantitative PCR. The biological activity of these transgenes was assessed by transformation in the bacterial strain *Acinetobacter* sp. BD413(pBAB2) in vitro. While the proportion of transgenes recovered increased with the increasing amount of transplastomic DNA added, plant DNA was rapidly degraded over time. The number of transgenes recovered decreased about 10,000 fold within 2 weeks. Data reveal, however, that a small fraction of the plant DNA escaped degradation. Transgene sequences were still detected after 4 years and transformation assays showed that extracted DNA remained biologically active and could still transform competent cells of *Acinetobacter* sp. BD413(pBAB2). The approach presented here quantified the number of transgenes (based on quantitative PCR of 50% of the gene) released and persisting in the environment over time and provided new insights into the fate of transgenic plant DNA in soil.
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1. Introduction

Horizontal gene transfer (HGT) is a fundamental mechanism of bacterial evolution and adaptation (Garcia-Vallve et al., 2000; Rensing et al., 2002), but becomes a source of concern if bacteria in the environment integrate and express specific non-indigenous genes from transgenic plants. Questions arise as to the possible impact of such transformation-mediated gene transfer if the newly acquired traits lead to increased fitness of these genetically transformed bacteria. Bacterial population shifts might occur, with the specific risk that the number of bacteria containing the newly acquired genes increases significantly.

While HGT between plants and bacteria has not been determined under natural field conditions, the spatial organization of plant-associated bacterial communities and the presence of plant tissues in soil could enhance such transfer. Soil is a heterogeneous matrix structured into microenvironments in which numerous and diverse soil bacteria develop microcolonies (Grundmann, 2004). Some soil bacteria possess a genetically encoded natural transformation potential (Demaneche et al., 2001a; Paget and Simonet, 1994) that can be expressed in situ (Demaneche et al., 2001a). Moreover, indigenous bacteria might be passively transformed when

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subjected to lightning discharges in situ (Cérémonie et al., 2004, 2006; Demaneche et al., 2001b). In addition, other chemical and physical conditions similar to those that caused the bacterium *Escherichia coli* to be genetically transformed in river water (Baur et al., 1996) might occur. The probability of transgene transfer to bacteria increases for transgenes containing prokaryotic sequences (Bertolla et al., 2000; de Vries and Wackernagel, 1998; Gebhard and Smalla, 1998; Kay et al., 2002). This suggests that the probability of HGT from plants to bacteria depends in part on the presence of homologous sequences in soil bacteria that would favor homologous recombination.

Based on gene transfer regulation, parameters such as the origin, transgene sequence and copy number of the transgene in the plant have a significant role in HGT. A significant percentage of DNA from decaying tobacco leaves escapes degradation and maintains its biological activity as shown by its ability to produce bacterial transformants in vitro (Ceccherini et al., 2003). Although several reports have shown that DNA released by plant material could persist in soil for months or years as extracellular material (Gebhard and Smalla, 1999; Paget et al., 1998; Widmer et al., 1996, 1997), little is known about the biological potential of this long-lasting DNA. Given that the greater the quantity of transgenic DNA released by the plant, the greater the potential for a soil bacterium to incorporate these sequences, transplastomic plants, which contain up to 10,000 copies of the transgene per cell (Bendich, 1987; Daniell et al., 1998), are particularly well suited for studying the fate of transgenes in soil and potential HGT between plants and bacteria.

The objective of this study was to investigate the physical persistence and biological activity (transformation frequency) of extracellular DNA either purified and added to soil or naturally released by decaying transplastomic tobacco leaves in soil. Soil microcosms were amended with purified plant DNA and stored at room temperature for up to 4 weeks before total DNA was extracted and quantified in Quantitative PCR assays and transformed into *Acinetobacter* sp. BD413 in vitro. The fate of plant DNA naturally released by transplastomic tobacco leaves in soil microcosms was similarly monitored for up to 210 weeks.

2. Materials and methods

2.1. Plant material

All experiments were performed using wild-type tobacco plants (*Nicotiana tabacum* cv. PBD6) and transplastomic plants of the same cultivar harboring an *aadA* gene, which confers resistance to both spectinomycin and streptomycin, inserted between the *rbcL* and *accD* plastid genes. The selectable marker gene *aadA* is driven by a chimeric rRNA operon promoter *Prrn* (Svab and Maliga, 1993). Description of the construction of the transplastomic plants has been reported previously (Kay et al., 2002).

2.2. Bacterial strains, plasmids, and culture media

The naturally transformable bacterium *Acinetobacter* sp. strain BD413 harbors the recombinant plasmid pBAB2 (Kay et al., 2002) in which plastid sequences corresponding to the *rbcL* and *accD* regions flanking the transgene have been cloned to facilitate homologous recombination with the transplastomic tobacco sequences. *Acinetobacter* sp. strain BD413(pBAB2) was used as the model strain for evaluation of the natural transformation potential of plant DNA. It was routinely grown at 28 °C on low salt Luria–Bertani medium (Bacto Tryptone, 10 g/l; yeast extract, 5 g/l; NaCl 5 g/l) (LB*m*) supplemented with ampicillin (50 μg/ml) and nalidixic acid (20 μg/ml) (Sigma, St. Louis, USA). Transformants were selected on LB*m* medium containing ampicillin (50 μg/ml), nalidixic acid (20 μg/ml), and spectinomycin (50 μg/ml). The population sizes of recipient and transformant cells were estimated from colony counts after 2 days of incubation on plates at 28 °C. *E. coli* DH5α, harboring the plasmid pCEA, was grown at 37 °C on LB*m* medium supplemented with ampicillin (50 μg/ml) and spectinomycin (50 μg/ml) (Sigma, St. Louis, USA). Plasmid pCEA (pLEP01) which had been used to transform tobacco plants (Kay et al., 2002), is a pBluescript® II SK+ derived, ampicillin-resistant cloning vector containing the *aadA* gene flanked with plastid sequences corresponding to part of the *rbcL* and *accD* regions (2.5 Kb) (Kay et al., 2002).

2.3. Soil microcosms

Microcosms consisted of non-sterile, sandy loam soil (50% sand; 41% clay; organic matter 40.6 g/kg of dry soil; pH 5.6) from La Côte Saint-André (Isère, France) in which intact or ground tobacco leaf tissue, or purified plant DNA (both nuclear and chloroplastic DNA), was added. Transplastomic and wild-type tobacco plant leaves were either cut into disks or ground in liquid nitrogen. A total amount of either 0.05 g or 0.5 g of plant material, corresponding to approximately a total number of 8×10^9 and 8×10^{10} *aadA* genes, respectively, was added to 10 g of soil. Control soil microcosms did not receive any plant material. Soil–plant mixtures were placed into 50 ml Falcon™ polypropylene tubes (Becton Dickinson, Franklin Lakes, USA) and maintained at room temperature and 10% humidity for up to 210 weeks. Similar experiments were performed by adding 1 ml of purified plant DNA solution. Transplastomic plant DNA was added to 10 g of soil in amounts ranging from 10^{-3} to 10^3 μg (10^{-3}, 10^{-2}, 10^{-1}, 10^0, 10^1, 10^2, and 10^3 μg) in 1 ml of ultrapure water. Mixtures were kept as described above for up to 4 weeks. Control soil microcosms were amended with 1 ml of sterile ultrapure water. Each experiment was performed in triplicate.

2.4. DNA extraction

Plant genomic DNA extraction from tobacco leaves was performed using the DNeasy® Plant Kit (Qiagen, Mannhein, Germany) according to the manufacturer's instructions.

Plasmid DNA was isolated from *E. coli* DH5α (pCEA) using the QIAfilter™ Plasmid Midi Kit (Qiagen, Hilden, Germany) following manufacturer's instructions. Total DNA was extracted from microcosms using the UltraClean™ Soil DNA Kit Mega Prep (Mo Bio Labs, Solano Beach, USA) following the manufacturer's instructions, 0, 2, 3, 4, and 210 weeks after addition of DNA to microcosms. DNA degradation was assessed by electrophoresis on 0.8% agarose gels. Purity of extracted DNA was assessed by PCR using primers pA and pH complementary to part of the 16S ribosomal DNA gene (Edwards et al., 1989). DNA concentrations were determined by measuring the absorbance of the solution at 260 nm (OD_{260}) with the Eppendorf® Biophotometer (Eppendorf, Westbury, USA).

2.5. Detection and quantification of transplastomic sequences

Detection and quantification of the transplastomic sequence signature were determined by PCR and quantitative PCR, respectively. Detection of transplastomic sequences in soil microcosms was performed by touchdown PCR, using published specific primers p1531cpl2up (′-TTTCTATTGTTGTCTTGGAT-3′) and p416 (5′-TGACGGGCTGATACT-3′) targeting a 853-bp sequence, and with primers (for q-PCR) p415 (5′-ATTCCGTGGCGTTAT-3′) and p416 (5′-TGACGGGCTGATACT-3′) complementary to part of the *aadA* gene and targeting a 382-bp fragment (Ceccherini et al., 2003). Denaturing and elongation steps were done at 95 °C and 72 °C, and the annealing temperature decreased by 2 °C during 10 cycles starting from 60 °C to 50 °C, followed by 25 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 1 min. The last cycle was done at 72 °C with a 7-min extension before storage at 4 °C. Transgene DNA sequences were quantified by quantitative PCR using the LightCycler® 1.5 system (Roche, Meylan, France). Quantitative PCR assays were carried out with 50 ng of template DNA using primers p415 and p416 to amplify a 382-bp fragment. The PCR reaction was adapted from Ceccherini et al. (2003) and consisted of an initial denaturation at 95 °C for 8 min followed with 45 cycles of 95 °C for 10 s, 55 °C for 8 s, and 72 °C for 16 s. Plasmid pCEA was used as a template to establish calibration curves and was serially diluted in distilled sterilized water at concentrations ranging from 3.2 ng/μL to 3.2×10^5 ng/μL. Data were analyzed with LightCycler® software 3.5.3 (Roche, Meylan, France). The number of *aadA* fragments in each sample was quantified by plotting the respective crossing-point value against standard concentrations. All samples were tested in triplicate, and the average value was used for quantification.

2.6. Natural transformation of Acinetobacter sp. BD413

The transformation potential of the extracted DNA was determined by transforming *Acinetobacter* sp. BD413 (pBAB2) with each DNA extract in vitro. An overnight culture of *Acinetobacter* sp. BD413(pBAB2) was diluted 25-fold with fresh LB*m* medium supplemented with the appropriate antibiotics and cultured for 2 h at 28 °C ($OD_{600} = 0.9$) to reach a competent state, as described by Palmen et al. (1993). Transformations were carried out by adding 12.6 μg, 1.4 μg or 1 μg of total DNA extracted from microcosms to 50 μl of ultrapure water, or 50 μl of serial dilutions of purified plant DNA (4, 0.4, 0.04, and 0.004 μg) to 250 μl of competent bacterial cells. The resulting mixtures were incubated for 2 h at 28 °C. Appropriate dilutions were then plated on LB*m* medium supplemented with the appropriate antibiotics. Population sizes of recipient and transformant cells were estimated from colony counts after 2 days of incubation at 28 °C. Three replicates were used for each sample of DNA. PCR assays, using primers p1531cpl2up and p416, were performed to confirm the presence of the transgene resulting from transformation by plant DNA in spectinomycin-resistant clones of *Acinetobacter* sp. BD413(pBAB2). To assess the effect of soil DNA on the transformation potential of *Acinetobacter* sp. BD413(pBAB2) by transplastomic plant DNA, different amounts of purified soil DNA and plant DNA were mixed and used to transform *Acinetobacter* sp. BD413 (pBAB2) as described above. DNA mixtures consisted of 0.8 μg of total DNA, where plant DNA represented 0.05%, 0.5%, 5%, 50%, and 100% of the total amount.

2.7. Data transformation and statistical analysis

Data transformation, estimation of the total number of *aadA* genes/μg of total DNA, descriptive statistics, statistical analyses and modeling of the fate of DNA in soil were performed using Microsoft Excel software (Microsoft Co., Redmond, USA).

3. Results

3.1. Release of DNA in soil from decaying plant material

Deterioration of plant tissue became visible within the first weeks after burial in soil. After 3 weeks, leaf disks appeared greenish, flaccid and water-soaked. Leaf disk degradation was more pronounced after 4 weeks in soil and, although the 0.5-g leaf disks remained intact, 0.05-g leaf disks were fragmented into small pieces. After 210 weeks, plant material incorporated into soil could not be detected visually. The quantity of total DNA extracted from soil microcosms where plant material was added increased significantly as a function of time. The average quantity of total DNA extracted from microcosms had doubled, on average, after 4 weeks. The amount of total DNA extracted after 210 weeks (5.5 ± 0.7 μg/g of soil) was slightly higher than that extracted after 4 weeks (4.8 ± 0.6 μg/g of soil), though not significantly different ($P = 0.34$). During the first 4 weeks, the amount of total DNA extracted from microcosms where ground leaf disks were added was more than 2-fold greater than that of microcosms where intact leaf disks of the same weight were added. This difference was less pronounced after 210 weeks: 5.9 ± 2.0 μg/g and 4.9 ± 1.0 μg/g were extracted from soil

microcosms where 0.5 g of ground and intact leaf disks were added, respectively.

3.2. DNA extraction from microcosms amended with purified DNA

The initial amount of total DNA extracted from control soil was 6.0 ± 5.7 μg/g of soil and did not differ significantly between experiments. The amount of total DNA extracted at time zero from microcosms amended with 10 μg or less of purified plant DNA/10 g of soil did not differ significantly from control soil and averaged 6.1 ± 1.2 μg/g of soil (Fig. 1). The quantity of DNA extracted at time 0 from microcosms amended with greater quantities of plant DNA were significantly greater than control microcosms and were 15.0 ± 1.4 μg/g of soil and 19.5 ± 3.0 μg/g of soil for microcosms amended with 100 μg and 1 mg of purified plant DNA/10 g of soil, respectively (Fig. 1). Although the average amount of total DNA extracted from microcosms increased over time, no significant differences were observed between microcosms with different amounts of plant DNA added initially. On average, 10.9 ± 1.3 and 9.3 ± 1.9 μg of DNA/g of soil were extracted from microcosms amended with purified plant DNA after 2 weeks and 4 weeks of incubation, respectively.

3.3. PCR detection of transgene sequences in soil

Transplastomic sequence signatures were successfully amplified by PCR performed with DNA solutions extracted from soil microcosms that had been amended with purified transplastomic plant DNA or plant tissue. DNA extracted from each soil microcosm was successfully amplified using primers complementary to highly conserved regions of the 16S rDNA (data not shown). Touchdown PCR assays performed with primers p415 and p416 and primers p1531cpl2up and p416 successfully amplified part of the *aadA* gene (382 bp) and a sequence containing part of the *aadA* gene and the adjacent plastid DNA region specific to the transgene (853 bp), respectively. Transplastomic plant DNA sequences were not amplified from total DNA solutions extracted from control microcosms.

After 4 weeks, transplastomic DNA sequences were only amplified from microcosms having been amended with 1 μg or more of plant DNA and only with the primer set p415 and p416 targeting the 382-bp fragment (Fig. 2). The expected 853-bp DNA fragment was amplified in all DNA extractions from soils amended with the transplastomic plant leaves and was absent from all soil microcosms where wild-type leaf tissue was added (Fig. 3). After 3 weeks, DNA extracted from microcosms that initially contained 0.5 g of intact or ground plant tissue yielded stronger band intensities than from microcosms amended with 0.05 g of leaf disks (Fig. 3A). The same trend was observed after 4 years for microcosms in which transplastomic DNA sequences were still amplified by PCR (Fig. 3B).

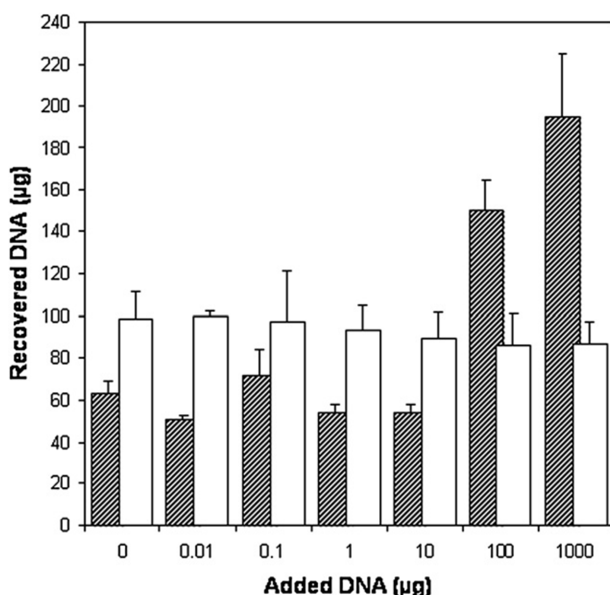

Fig. 1. Total amount of DNA extracted as a function of the amount of purified plant DNA added to soil (10 g) microcosms immediately after addition (shaded bars) and after 4 weeks in soil (white bars). Error bars represent the standard error of the mean amount of DNA recovered.

3.4. Quantitative PCR quantification of transgene sequences in soil

Quantitative PCR results confirmed those obtained with touchdown PCR with the detection of transgene sequences after up to 210 weeks and only in soil samples amended with transplastomic leaves or purified transplastomic DNA. The number of *aadA* genes/μg of plant DNA (determined by quantitative PCR) added to soil microcosms was $1.3 \pm 0.2 \times 10^9$ *aadA* genes/μg of plant DNA, which corresponded to about 7000 copies of the transgene/plant cell. Regardless of the initial amount or form of transplastomic DNA added to microcosms (i.e. purified, intact, or ground tissue), the kinetics of degradation of plant DNA in microcosms showed that the number of *aadA* gene fragments decreased dramatically over time (Fig. 4). The total number of *aadA* gene fragments decreased by over 10,000 fold within the first 2 weeks and by only 10-fold during the following 207 weeks of the experiment. No significant differences in the number of *aadA* gene fragments were observed between microcosms amended with the same amount of plant DNA as either purified DNA or intact and ground leaf tissue at any time point. The number of *aadA* gene fragments amplified from soil amended with 0.5 g of leaf disks was about 10-fold larger ($3.1 \pm 0.2 \times 10^6$) than that of soil microcosms amended with 0.05 g of leaf disks ($3.3 \pm 0.1 \times 10^5$) after 4 weeks. After 210 weeks, *aadA* fragment sequences were amplified from microcosms amended with 0.5 g of leaf disks but not from those amended with 0.05 g of leaf disks (Fig. 4).

3.5. Fate of transplastomic plant DNA released in soil

The fate of transplastomic plant DNA added to soil microcosms was determined as a function of time. The transgene was

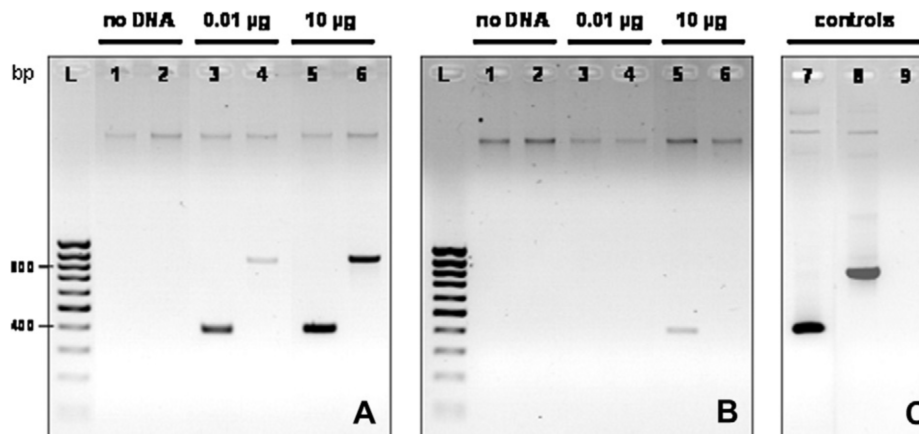

Fig. 2. Amplification of transplastomic plant DNA sequences extracted from 10 g soil microcosms immediately after addition of purified plant DNA (A) and after 4 weeks in soil (B) visualized by agarose gel electrophoresis. Detection of transplastomic sequences in soil microcosms was performed by PCR using 2 primer sets targeting 382-bp (left) and 853-bp (right) fragments, respectively. DNA extracted from non-amended microcosms (1, 2), from microcosms amended with 0.01 µg of purified plant DNA (3, 4), or with 10 µg of purified plant DNA (5, 6) are shown. (C) Positive controls performed using purified plasmid DNA as a matrix (7, 8) and negative control, H_2O (9).

quantified by quantitative PCR. Data were normalized and expressed as the number of *aadA* gene fragments divided by the total amount of DNA. Regardless of the amount of transplastomic plant DNA added to soil microcosms, the number of transgenes recovered decreased rapidly over time, thus confirming the data presented previously. The presence of the transgene could not be detected in microcosms amended with 0.1 µg or less of purified plant DNA after 0 and 2 weeks or in microcosms amended with 1 µg or less after 4 weeks (Fig. 5). The percentage of transgenes recovered immediately after addition of purified plant DNA to microcosms was, on average, about 2% of the number of transgenes added. No significant differences were observed in the amount of transgenes recovered after 4 weeks from microcosms amended with plant tissue or with an equivalent amount of purified plant DNA. Nonetheless, the proportion of transgenes recovered was roughly proportional to the number of transgenes added to the soil microcosms and increased with increasing amounts of transplastomic DNA added (Fig. 5). For example, immediately after the addition of plant DNA (and also after 2 weeks), the percentage of transgenes recovered from microcosms amended with 10^3 µg of purified plant DNA was about 10-fold greater than that of microcosms amended with 1 µg of purified plant DNA (the addition of 1 µg of plant DNA corresponded to approximately 10^7 *aadA*/µg of total DNA).

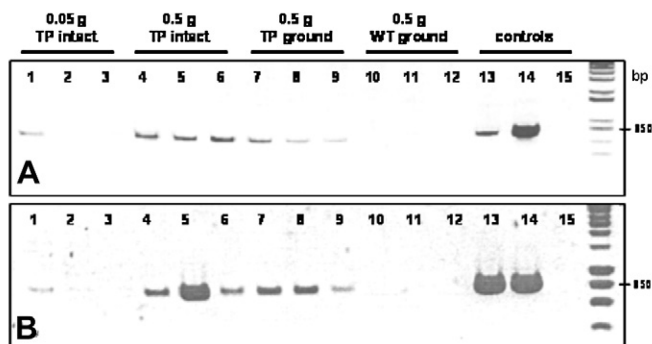

Fig. 3. Amplification of transplastomic plant DNA sequences extracted from 10 g soil microcosms amended with leaf disks after 3 weeks (A) and 210 weeks (B) visualized by agarose gel electrophoresis. Detection of transplastomic sequences was performed by PCR using primers p1531cpl2up and p416 targeting a 853-bp fragment. DNA extracted from microcosms amended with 0.05 g intact leaf disks (1–3), with 0.5 g intact leaf disk (4–6), with 0.5 g ground leaf disks (7–9) of transplastomic tobacco plants (TP), and with 0.5 g ground leaf disks of non-transplastomic tobacco plants (WT); Purified plasmid DNA (pCEA, 16 ng) (13); purified transplastomic plant DNA (30 ng) (14); negative control (H_2O) (15).

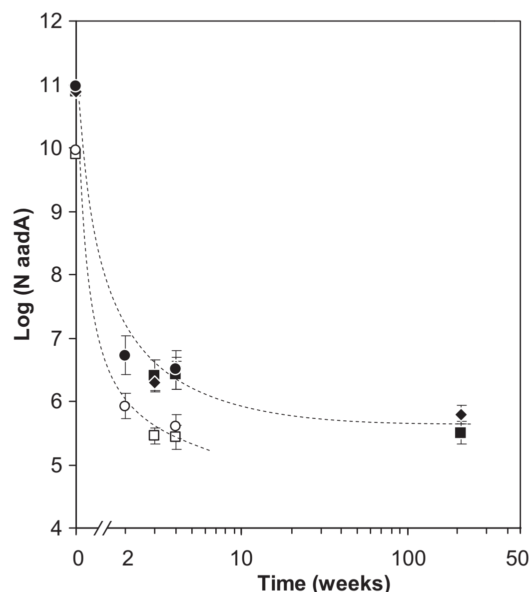

Fig. 4. Kinetics of plant DNA degradation in soil microcosms as estimated by the number of *aadA* gene fragments amplified by quantitative PCR. Different amounts of purified plant DNA were added to soil microcosms (10 µg, white circle; 100 µg, black circles), and degradation was monitored for up to 4 weeks. The number of *aadA* gene fragments in soil microcosms where intact 0.05 g (white squares) and 0.5 g (black squares) of leaf disks and 0.5 g of ground leaf disks (black diamonds) were added was quantified for up to 210 weeks. Dotted lines represent best fit of the equations. Vertical bars represent the standard error of the mean number of *aadA* gene fragments amplified.

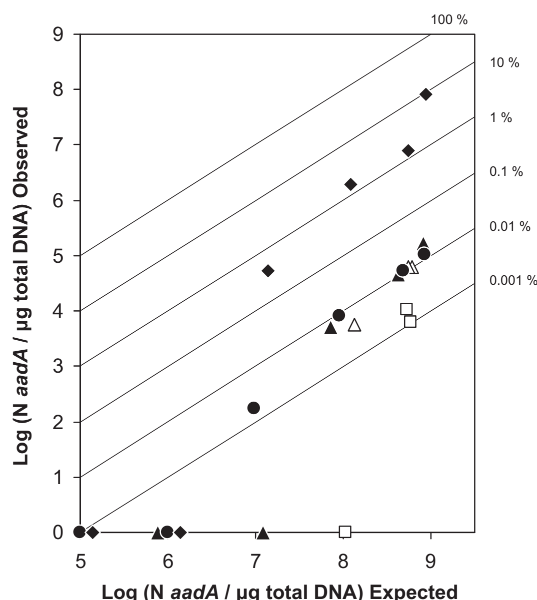

Fig. 5. Number of *aadA* gene fragments recovered as a function of the number of *aadA* genes added to soil microcosms. Data were normalized and expressed as the number of *aadA* gene fragments divided by the total amount of DNA extracted. Quantification was performed after 0 (diamonds), 2 (circles), 4 (triangles), and 210 weeks (squares) for purified plant DNA (filled) or leaf disks (open) in the soil microcosms. The addition of 1 µg of plant DNA corresponded to approximately 10^7 *aadA*/µg of total DNA. Dotted lines correspond to the relative percentage of *aadA* gene fragments recovered and amplified by quantitative PCR. Standard error bars were removed for clarity but averaged 0.25 ± 0.08 log(*N aadA*/µg total DNA).

Degradation of plant DNA increased rapidly with time, and 2.3, 0.01, 0.01, and 0.002% of added DNA were recovered after 0, 2, 4, and 210 weeks of incubation, respectively. Although the DNA extraction yield remained an unknown variable, most likely resulting in an overestimation of the amount of DNA degraded or adsorbed, the amount of plant DNA recovered after 2 weeks and 210 weeks was, on average, 200-fold and 1200-fold smaller, respectively, than that recovered immediately after addition of plant DNA to soil microcosms.

3.6. Transformation by plant DNA extracted from soil

Transplastomic plant DNA present in soil for up to 210 weeks remained biologically active and still transformed competent cells of *Acinetobacter* sp. strain BD413(pBAB2) in vitro. Transformants of the recipient strain were obtained only with soil samples at time 0 (i.e. immediately after addition of purified DNA to soil microcosms) and not after 2 weeks and 4 weeks. On the other hand, transformants were obtained using total soil DNA extracted from soil samples amended with 0.5 g ground leaf disks after 3 and 210 weeks, but not with any of the microcosms amended with only 0.05 g leaf disks as either ground or intact tissue. PCR amplifications and sequence analysis confirmed that the spectinomycin-resistant clones obtained resulted from transformation of plant DNA extracted from microcosms. Transformation frequencies obtained in vitro with transplastomic plant DNA extracted from soil microcosms were similar to those obtained with purified plant DNA and were proportional to the number of transgenes present in the DNA extracts used to transform *Acinetobacter* sp. (Fig. 6). The slopes of the linear regressions were 0.57 and 0.55 for transformation assays performed with purified DNA and extracted DNA, respectively. Transformation frequencies obtained with DNA extracted from soil were slightly lower (by about 2.8-fold) than those obtained with purified plant DNA (Fig. 6). To assess the effect of soil DNA on the transformation potential of *Acinetobacter* sp. by transplastomic plant DNA, different amounts of purified soil DNA (DNA extracted and purified as described in Section 2) and plant DNA were mixed together and used to transform *Acinetobacter* sp. BD413(pBAB2). Transformation efficiencies were weakly affected by the presence of non-target DNA or soil impurities and were a function of the number of transgenes. For example, when 400 ng of purified plant DNA was mixed with 400 ng of soil DNA, transformation frequencies were, on average, 2.6-fold lower than those obtained with purified plant DNA only and were (expressed as log[transformation frequencies]) -5.3 ± 0.5 and -5.7 ± 0.5 for plant DNA and plant DNA mixed with soil DNA, respectively.

4. Discussion

Investigation of the physical persistence and biological activity of extracellular plant DNA amended or naturally released by decaying transplastomic tobacco leaves in soil

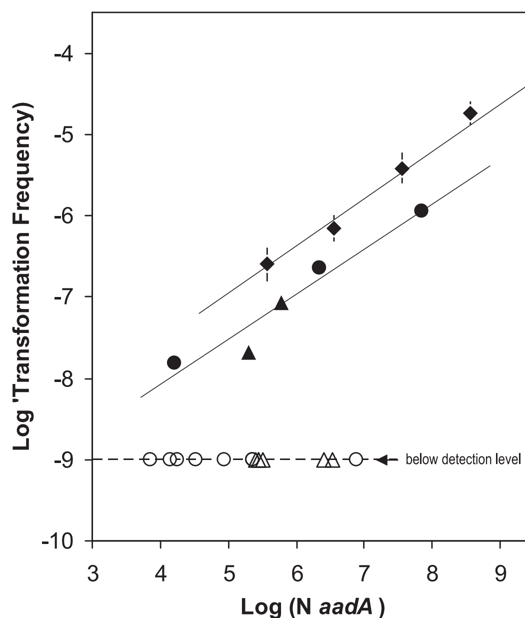

Fig. 6. In vitro transformation of *Acinetobacter* sp. BD413(pBAB2) with purified plant DNA (diamonds) and with DNA extracted from soil microcosms amended with purified plant DNA (circles) or leaf disks (triangles). Transformation frequencies are expressed as the number of transformants/number of recipient cells. Open symbols correspond to transformation frequencies that were below the detection level ($\sim 10^{-9}$). Dashed lines represent the linear regression of the transformation frequency of *Acinetobacter* sp. BD413 (pBAB2) as a function of the number of *aadA* genes present in the solution for purified plant DNA ($y = 0.57x - 9.85$, $R^2 = 0.986$) and for DNA extracted from soil ($y = 0.55x - 10.29$, $R^2 = 0.942$).

revealed that DNA sequences can persist in soil and remain biologically active for several months. The approach used to assess the amount of specific DNA sequences persisting in soil included direct extraction of total DNA, its purification and the use of specific primers to amplify target DNA by PCR. While the quantitative PCR method used could detect as few as 10^2 copies of the transgene per μg of soil DNA, methodological biases could, however, lead to an underestimation of the actual number of DNA sequences present in soil. For example, precise DNA extraction yields, which remained undetermined, or the dilution of target DNA sequences among the total genomic DNA (the metagenome) could have biased the PCR amplification process, leading to an underestimation of the actual number of specific targets. In spite of these potential methodological biases, the number of transgenes amplified was systematically lower after 2 and 4 weeks than at time 0. The drop was greater within the first 2 weeks (up to 3 orders of magnitude) than between weeks 2 and 4, for which the number of transgene and the amount of DNA recovered were similar. Measured DNA remaining after 2 and 4 weeks most likely belonged to the fraction that was neither irreversibly adsorbed initially onto soil components nor subjected to rapid degradation by nucleases naturally present in soil. We hypothesized that the reduced rate of DNA degradation with time might result from an increasing spatial segregation of DNA and the microorganisms responsible for its degradation in the soil matrix as the DNA nearby would be increasingly degraded. Perturbation of the soil by mixing or adding water would most likely result in an increased degradation rate of the remaining plant DNA. This experiment was not performed so the hypothesis remains untested.

DNA adsorbs tightly to soil components such as clay particles or sand, and therefore, DNA recovery rates can be low and even less than 1% for clay-rich soils (Frostegard et al., 1999). DNA quantities were probably underestimated for microcosms amended with the lowest amount of DNA. However, designing experiments to quantify degradation kinetics of adsorbed DNA in soil, its availability for bacteria and its possible desorption remains a methodological challenge. According to previous reports in which DNA adsorption potential was tested with clay particles, most of the adsorbed DNA remains accessible to both nucleases and bacteria while the rest was efficiently protected against degradation and unavailable for competent bacteria (Demaneche et al., 2001c). In our study, a fraction of the adsorbed DNA might have remained accessible to degradation, but the adsorption of DNA onto soil components may also increase with decreasing DNA fragment size, which results from the degradation of free DNA.

Plant DNA is thought to be released gradually from decaying tissue (Ceccherini et al., 2003), most likely leading to DNA adsorption and degradation that cannot be satisfactorily simulated by amending soil with pure plant genomic DNA. Developing and using soil microcosms in which plant material was left to decay was a further step to simulate the release of DNA under natural conditions in order to assess the persistence of transforming DNA. Surprisingly, the number of transgenes amplified after 2-week incubation or more did not differ from that amplified from microcosms amended with a comparable amount of purified plant DNA. While purified plant DNA might have been degraded more rapidly in soil than the plant DNA contained in leaf tissue, no differences in the number of *aadA* gene fragments were observed after 2 weeks. These observations suggest that degradation of DNA in soil, regardless of its state (purified or within plant tissues), occurs rapidly. While purified DNA may have been degraded more rapidly, DNA extractions performed after only 2 weeks may have been too late to observe a difference in the degradation kinetics of purified plant DNA and DNA inside plant tissues. In previous studies, the DNA released from decaying tobacco leaves was degraded within 72 h (Ceccherini et al., 2003). Both total DNA and *aadA* gene numbers decreased drastically within 72 h, which suggests that plant nucleases were active and that the shearing process that decreases the average size of plant DNA fragments may prevent amplification of *aadA* sequences. Bacterial cell debris may also protect DNA from inactivation in soil (Nielsen et al., 2000). While this beneficial effect might apply to plant material, plant nucleases may counteract the protective effect of plant cell debris and account for the rapid degradation of plant DNA observed in soil.

In addition to the quantification of transgene persistence in soil, the biological potential of extracellular DNA was considered. Most extracellular DNA in soil may remain undetected, yet some could be involved in a genetic transformation process with indigenous soil bacteria. Assessment of DNA availability and transformation potential by inoculating soil with recipient bacteria to trap DNA before isolating transformants suffers major flaws. Inoculated bacteria colonize a small proportion of the soil, often limited to the outer soil compartments without penetrating soil micro-aggregates (Recorbet et al., 1995), thus avoiding most of the indigenous extracellular DNA. Moreover, naturally transformable bacteria are transformed at very low frequency in situ due to a rapid loss of competence ability in soil (Nielsen et al., 1997). Since testing transformation directly in soil seems unsatisfactory for many reasons, tests were developed to determine transformation efficiency of a recipient strain submitted in vitro to DNA extracted from soil. Controls were carried out with pure transplastomic plant DNA in the presence, or not, of soil contaminants and indigenous DNA. We observed that transformation efficiency of extracted DNA was related to the number of target genes in the DNA and was slightly lower than that of pure plant DNA (Fig. 6). Purified DNA added to soil and immediately extracted without any incubation exhibited transformation efficiency lower than pure DNA for the same number of PCR target genes. These results suggest a discrepancy between the presence of the transgene as detected by quantitative PCR amplification and the biological potential of these DNA molecules to transform bacteria. The addition of DNA to soil for no more than a few seconds might have been too brief for soil nucleases to degrade DNA, but long enough for chemical alteration to reduce transformation efficiency. DNA extracted from soil samples amended with leaf disks for 2, 4 or 210 weeks had the same discrepancy

between quantitative PCR calculated number of target genes and the corresponding transformation efficiency. Such differences may result from the presence and effect of inhibitory compounds on extraction and PCR amplification yields. On the other hand, quantification of *aadA* genes by quantitative PCR using primers targeting only part of the *aadA* gene (382 bp) may lead to an overestimation of the actual number of functional *aadA* gene fragments that could transform *Acinetobacter* sp. strain BD413. Indeed, amplification of a subfragment of the transgene does not necessarily imply that the entire transgene and flanking regions long enough to allow recombination in the recipient strain were present. That could explain why transformants were not obtained with some of the DNA extracts containing a priori a significant number of transgenes (Fig. 6). Such hypotheses could also explain the difference observed in the transformation potential of DNA extracted from microcosms amended with purified DNA and those amended with leaf disks. Transformants were obtained with soil samples amended with purified plant DNA at the sampling time zero only, while transformants were obtained with DNA extracted after 3 and 210 weeks from microcosms amended with leaf tissue. PCR amplification of the transgene as shown in Figs. 2 and 3 would confirm this hypothesis; after 4 weeks only 382-bp fragments were amplified with DNA extracted from microcosms amended with purified DNA, yet, 853-bp fragments were still present after 210 weeks in microcosms amended with leaf tissue. We hypothesize that plant DNA gradually released from decaying plant tissue was not as degraded as purified DNA, and thus, for the same number of *aadA* gene fragments amplified by quantitative PCR, the number of functional *aadA* genes (i.e. larger DNA fragments) is more abundant in microcosms amended with leaf tissue. While plant DNA is rapidly degraded by plant nucleases during the decaying process, cell debris may play a protective role and allow plant DNA to persist and remain biologically active in soils for long periods. During the first two weeks of the experiments, the half-life of plant DNA added to soil was estimated to be 23 h, which implies that after about 40 weeks, no transgene would be amplified from soil. However, the transgene was detected for up to 210 weeks, with a half-life of 83 days when long-term data (longer than 2 weeks) was used.

While determination of transgene copies in extracted DNA may not provide exact data about DNA availability for bacteria in situ, such an approach most likely underestimates the gene pool that could be involved in a transformation process. We reported here that the physical persistence and biological activity of extracellular plant DNA released by decaying transplastomic tobacco leaves in soil lasts for up to 4 years. The low number of transgenes amplified after two weeks revealed that plant DNA was rapidly degraded. Although persistence and biological activity of plant DNA in soils is long-term, the direct contact between soil bacteria and plant DNA most likely occurs during the first days of plant tissue degradation. We hypothesize that the presence of degrading plant tissue in soil may (i) promote the growth of soil bacteria or bacteria naturally occurring on plant tissue by providing a novel and abundant source of nutrients, (ii) lead to a localized copiotrophic environment potentially fostering cell competence and (iii) favor direct contact between bacterial cells and plant DNA. Therefore, while plant DNA may persist in soils for long periods, horizontal gene transfer and acquisition of the transgene by soil bacteria is most likely to occur during the initial plant tissue degradation in soils, when plant DNA is still abundant and bacterial cells actively growing.

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